Intrinsic Patterns of Behavior of Epithelial Stem Cells

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The early concepts concerning hematopoietic and epithelial stem cells that were derived from kinetic studies have been greatly enhanced by new information about a range of other properties of somatic and embryonic stem cells. Firstly, the stem and amplifying pattern characteristically established by epithelial lineages has been found to represent an intrinsic pattern that is generated by somatic epithelial stem cells without the need for additional environmental information. Secondly, it is now apparent that somatic epithelial stem cells are plastic and can be directed into a range of new pathways of differentiation by heterotypic interactions. The mechanisms of this plasticity need to be reconciled with the normally stable commitment of these cells to production only of progeny entering a tightly restricted range of phenotypic pathways. The present review discusses the intrinsic properties of epithelial stem cells and how they may be acted upon by connective tissues to generate a wide range of phenotypically different epithelial structures.

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In renewing tissues such as stratifying squamous epithelia, the mechanisms controlling cell proliferation, differentiation and death are of central importance to the maintenance of normal structure, to physiological repair, and to a range of neoplastic and other pathological changes. The early view that basal cells are equipotential, and either proliferate or differentiate as chance events, is incompatible with the patterns of mitosis since found in epithelia and it is now clear that basal cells are heterogeneous in regard to their proliferative capacities (Leblond *et al*, 1964). The concept of epithelial stem cells was first proposed to explain the small relatively simple proliferative units found in rodent epidermis (Mackenzie, 1969, 1970; Potten, 1974) and it has since become apparent that similar proliferative hierarchies of stem, amplifying and post-mitotic differentiating cells are associated with the renewal of most, perhaps all, epithelia (Lavker and Sun, 1982; Potten, 1983; Hall and Watt, 1989; Cotsarelis *et al*, 1999). Basic defining features initially proposed for stem cells included a high capacity for self-renewal and an ability to produce cells that differentiate to maintain tissue function (Lajtha, 1979). The validity of these concepts for epithelial stem cells has now been supported by a wide range of studies, but these studies have also shown that the behavior and distribution of stem cells can differ quite markedly between various types of epithelia (Cotsarelis *et al*, 1999; Lavker and Sun, 2000; Alonso and Fuchs, 2003; Fuchs and Watt, 2003).

Stem cells in murine epithelia were anticipated to be slowly cycling (Potten, 1974) and this property enabled their initial localization in tissues by "label retention", a method that involves labeling epithelia *in vivo* with tritiated thymidine and sampling tissues a month or more later when division had diluted label from cycling cells (Bickenbach, 1981). "Label-retaining cells" (LRC) in murine epithelia have a range of features supporting their identity as stem cells (Bickenbach, 1981; Mackenzie and Bickenbach, 1985). For example, they are found beneath the central regions of columnar units in epidermis, at the deep tips of epithelial rete, and at the base of the anterior and posterior columns of tongue papillae, all sites predicted to be stem cell locations by other kinetic studies (Hume, 1983; Potten, 1983). Further evidence comes from demonstration that LRC are more clonogenic than other cells (Morris and Potten, 1994) and from lineage studies suggesting that the units of structure in murine epidermis are clonal, each being renewed by a single stem cell (Mackenzie, 1995; Ghazizadeh and Taichman, 2001). For more complex epithelial structures the patterns of stem cell distribution are not yet fully clear but LRC have been localized to the "bulge" region of hair follicles and to the limbal region of the cornea, regions shown to contain cells with high clonogenic potential (Cotsarelis *et al*, 1989, 1990; Rochat *et al*, 1994). In human epidermis, which is thicker than rodent epidermis, patterns of staining for β1 integrins, combined with staining for markers of proliferation and differentiation, indicate that stem cells lie among the basal cells in zones overlying the tips of the connective tissue papillae (Jensen *et al*, 1999) but the number of stem cells in these locations is uncertain.

The behavior of epithelial stem cells is of significance to a broad range of clinical problems. For example, interest in stem cell properties has arisen from their predicted roles in the initiation and progression of malignancy (Morris, 2000) and particularly how the persistence of stem and amplifying patterns in malignancy would influence the behavior of malignant lesions and their responses to therapy (Denekamp, 1994; Kummermehr, 2001).

Further interest in somatic stem cells has been stimulated by the need for stem cell transduction in gene therapy and by the requirements for stem cell manipulation during tissue engineering procedures (Bickenbach and Roop,

1999; Bickenbach and Dunnwald, 2000). Surprisingly, it has now been shown that a range adult somatic stem cells, including those for keratinocytes, can be induced by appropriate developmental signals to participate in the formation of a much wider range of tissues than had previously been believed (Liang and Bickenbach, 2002; Prockop, 2002). One of the aims of tissue engineering is to amplify stem cells and direct their progeny into patterns of differentiation and morphogenesis appropriate to the regeneration of new tissues. We have been particularly interested in two questions of relevance to these processes: (a) the extent to which somatic stem cell behavior is intrinsically determined or is dependent on interactions with the environment and (b) the degree to which the particular patterns of differentiation acquired by stem cell progeny are determined by a pre-existing commitment of the somatic stem cells producing them. In the present review, we discuss experimental work related to these topics and we conclude from current evidence that; (a) that the basic stem and amplifying pattern associated with epithelial renewal is an intrinsic epithelial property; (b) somatic stem cells are normally committed to generate progeny that enter restricted pathways of differentiation; (c) mesenchymal signals can direct stem cell progeny into new pathways of differentiation; and (d) mesenchymal modulation of the basic stem and amplification pattern is required to produce and maintain most epithelial structures.

Intrinsic Stem Cell Behaviors

As it is not possible to undertake *in situ* labeling studies of human epidermis, information about human epithelial stem cells has been derived mostly from *in vitro* studies, primarily those assaying the "stemness" of cells as measured by clonogenicity. The widely differing proliferative potentials of keratinocytes isolated from normal human epithelia can be demonstrated by plating them for growth at clonal densities (Barrandon and Green, 1985, 1987). Some cells give rise to round colonies composed of small compact cells that can be repeatedly passaged; others form irregular colonies capable of less extensive growth; yet others form colonies of large flattened cells that do not passage well. These colony forms, referred to as holoclones, meroclones and paraclones, are thought to be derived from, and consist of, stem cells and early and late amplifying cells, respectively (Barrandon and Green, 1987). The ability, however, of normal human keratinocytes to grow *in vitro* is limited and after extensive passage all colonies begin to assume the appearance of paraclones and cease division, a change that appears to be associated with depletion of stem cells. For example, when human keratinocytes marked by retroviral transduction are mixed with unmarked keratinocytes, the patterns of clonal extinction observed at late passages suggest that very few cells are functioning as stem cells and that the cultures consist essentially of amplifying cells (Mathor *et al*, 1996). It is therefore interesting that, after their *in vitro* expansion, transplantion of epidermal and mucosal keratinocytes to *in vivo* sites results in reformation and maintenance of a functional epithelium for many years (De Luca *et al*, 1990; Compton *et al*, 1998). Similar conclusions can be drawn from studies dealing with the characterization, culture, and transplantation of limbal stem cells for corneal regeneration following chemical burns and other injuries (Rama *et al*, 2001; Espana, 2003). Cell cultures thus seem to contain cells that are capable of maintaining or regaining the stem cell function of indefinite renewal when returned to the *in vivo* environment. An understanding of the way that the *in vivo* and *in vitro* environments differ in their ability to maintain stem cells appears central to further development of many of the tissue-engineering procedures now being proposed. The present inability to identify and examine directly the fate of human epithelial stem cells *in vitro* hinders investigation of these mechanisms.

One way that the *in vitro* and *in vivo* environments differ is that epithelia normally exist in association with mesenchyme and both the initial development of epithelial structure and its maintenance throughout adult life depend on interactions between epithelium and mesenchyme (Cunha *et al*, 1985; Mackenzie, 1994). Work with compound cultures has enabled analysis of interactions occurring between adult cells and a series of interesting experiments (Maas-Szabowski *et al*, 1999; Maas-Szabowski *et al*, 2001) has shown that reciprocal signals are generated by paracrine acting cytokines; e.g., keratinocyte growth factor is released by fibroblasts in response to interleukin 1 production by the epithelium. These interactions are necessary to maintain good patterns of growth and differentiation of epithelia. The general pattern is that regionally different keratinocytes grown on plastic retain some phenotypic differences, grown as organotypic cultures they express good regional differentiation patterns and, typically, when returned to an *in vivo* environment, fully regain their original phenotype. Such phenotypic stability is the basis of current tissue-engineering procedures where, after *in vitro* generation and transplantation of human epidermis, the particular phenotype of the epidermal region of origin remains expressed for years (Compton *et al*, 1998). The commitment of somatic epithelial stem cells to regionally various differentiation pathways thus seems to be quite rigid but it has been experimentally demonstrated that epithelia can be directed into new phenotypic patterns under the influence of regionally differing embryonic, and even adult, mesenchymes (Mackenzie, 1994). In response to embryonic developmental signals, somatic stem cells from skin can give rise to an even wide range of new lineages (Liang and Bickenbach, 2002). Information about the nature of these signals, and about how the progeny of somatic stem cells can be directed into particular differentiation pathways, is becoming of major interest to tissue engineering (Prockop *et al*, 2003).

In Vitro Behavior of Murine Keratinocytes

Clonal growth of murine epidermal keratinocytes *in vitro* initially required complex growth media together with feeder cells (Morris *et al*, 1987) but simpler media that permit expansive clonal growth in the absence of feeder cells have now been reported (Hager *et al*, 1999; Caldelari *et al*, 2000). Using a modification of the methods of Caldelari and coworkers, we have been able to grow and extensively passage keratinocytes isolated from a range of murine skin and

mucosal tissues. We have found that murine keratinocytes, like human keratinocytes, show varying clonogenic abilities, but that they differ from human keratinocytes in the morphologies and behaviors of the colonies they produce.

Figure 1 illustrates the series of morphologically distinct colony types formed by murine keratinocytes when they are isolated and plated at clonal densities. We have termed these colonies Type I, II, and III in reference to the number of cell types they contain. Type I colonies consist only of large flattened (Type 1) cells and show little growth (Fig 1*C*). Type II colonies consist of smaller relatively uniform (Type 2) cells surrounded by a peripheral zone of cells corresponding to the larger flattened Type 1 cells (Fig 1*B*). Type III colonies have an additional central zone consisting of small and closely packed (Type 3) cells (Fig 1*A*). The boundaries established between these zones of differing cells are usually quite well delineated (Fig 1*N*). On repeated bulk passage these cell morphologies and colony forms are recapitulated but if the cells of individual Type I, II, and III colonies are individually isolated and plated for growth, it is found that only Type III colonies contain cells that are capable of expansive growth and reformation of new Type III colonies. The clonogenic potential required to support extensive passage is thus restricted to the central Type 3 cells. Despite differences of these colony forms from those developed by human keratinocytes, particularly in the concentric distribution of cells in Type III colonies, it appears that the Type I, II, and III colonies are the functional murine equivalents of, respectively, the paraclone, meroclone, and holoclone forms of human colonies. These mutine colony morphologies are developed by keratinocytes from both epidermis and mucosa with functional stem cells apparently restricted to the Type III colonies. Some additional support for this notion has been provided by other findings. For example, the central Type 3 cells in colonies developed from oral mucosa fail to stain for cytokeratin 6 (Fig 1*D*), a marker of early differentiation in murine mucosal cells (unpublished observations), but stain positively for cytokeratin 15 (Fig 1*E*), a putative stem cell marker both in hair (Lyle *et al*, 1999) and human oral mucosa (Fig 1L). There appear to be two distinct mechanisms by which expansion of the population of Type 3 cells is controlled. Firstly, time-lapse video recording shows that proliferation of Type 3 cells results in centrifugal movement and transition into Type 2 cells. Secondly, morphological observations and staining for Caspase 3 indicate that apoptosis occurs relatively infrequently among the Type 2 cells but quite frequently among the Type 3 cells of the central region (Fig 1*F, G*). There are few data concerning differential apoptotic sensitivities of stem and amplifying cells of stratifying epithelia but possibly sensitivity of these cells to apoptotic stimuli may reflect the similar sensitivity of stem cells of some regions of the gut (Potten *et al*, 2003).

The Ability of Stem Cells to Generate Clonal Units of Structure *In Vivo*

The clonal patterns developed *in vitro* by isolated human and murine keratinocytes indicate the intrinsic nature of their ability to generate stem and amplifying patterns. Lineage studies of murine epidermis indicate that individual stem cells generate clonal units *in vivo* and that these correspond morphologically to the small columnar units normally present (Mackenzie, 1995; Ghazizadeh and Taichman, 2001). To test whether establishment of this *in vivo* clonal pattern also represents an intrinsic ability of epidermal stem cells, epidermal keratinocytes were recombined with connective tissues from regions without columnar epithelial. The reformation of clonal columnar units in these preparations indicated that the epidermal pattern can be regenerated in the absence of regionally specific mesenchymal signals (Mackenzie, 1995). We have since extended these types of lineage studies to human epithelia by transduction of epidermal keratinocytes to produce populations containing a mixture of marked and unmarked cells. These were then used to generate organotypic cultures for transplantation to SCID mice for up to 12 wk. The results obtained with human epidermis were similar to those for murine epidermis. Two wk after transplantation, the reformed epidermis was hyperplastic and contained large irregular clusters of labeled cells that appeared to be differentiating but, by 12 wk, hyperplasia had diminished and both the number and size of the labeled cell clusters was greatly reduced (Fig 1*H*). The labeled cell clusters that remained corresponded morphologically to the columnar units present in human epidermis (Mackenzie *et al*, 1981) suggesting that thin human epidermis, like mouse epidermis, consists of small clonal units of structure.

Stem Cell Lineages in Larger Epithelial Structures

The small clonal units found in rodent epidermis are not typical of most epithelia. For example, stem cells in hair follicles are restricted to the bulge region, stem cells in cornea are found in the surrounding limbal region, and stem cells in gut lie at the base of the villi (Cotsarelis *et al*, 1989, 1990; Potten and Loeffler, 1990). Even in fairly simple epithelial structures, such as rete ridges, stem cells appear to be restricted to certain zones but, strangely, the position of such zones differs between skin and mucosal epithelia: In the epidermis, stem cells are clustered over the tips of the connective tissue papillae (Jensen *et al*, 1999) but lie at the deep tips of the epithelial rete of palmar epidermis (Lavker and Sun, 1982) and in mucosal epithelia (Seery and Watt, 2000). With the aim of seeing whether additional information about these patterns could be obtained by lineage studies, human keratinocytes and fibroblasts were isolated from palate or gingiva, oral regions that have thick keratinized epithelia with a regular rete pattern (Schroeder, 1981). Following labeling, reconstruction as organotypic cultures, and transplantation to SCID mice, these keratinocytes reformed quite thick epithelia with an undulating epithelial-connective tissue interface and rete-like structures. The clonal cell lineages developed in these tissues took the form of columns of cells running through the full epithelial thickness but they were narrower than the rete structures and bore no fixed relationship to them (Fig 1*I*). This was an unexpected finding as we had anticipated that palatal stem cells would lie at the

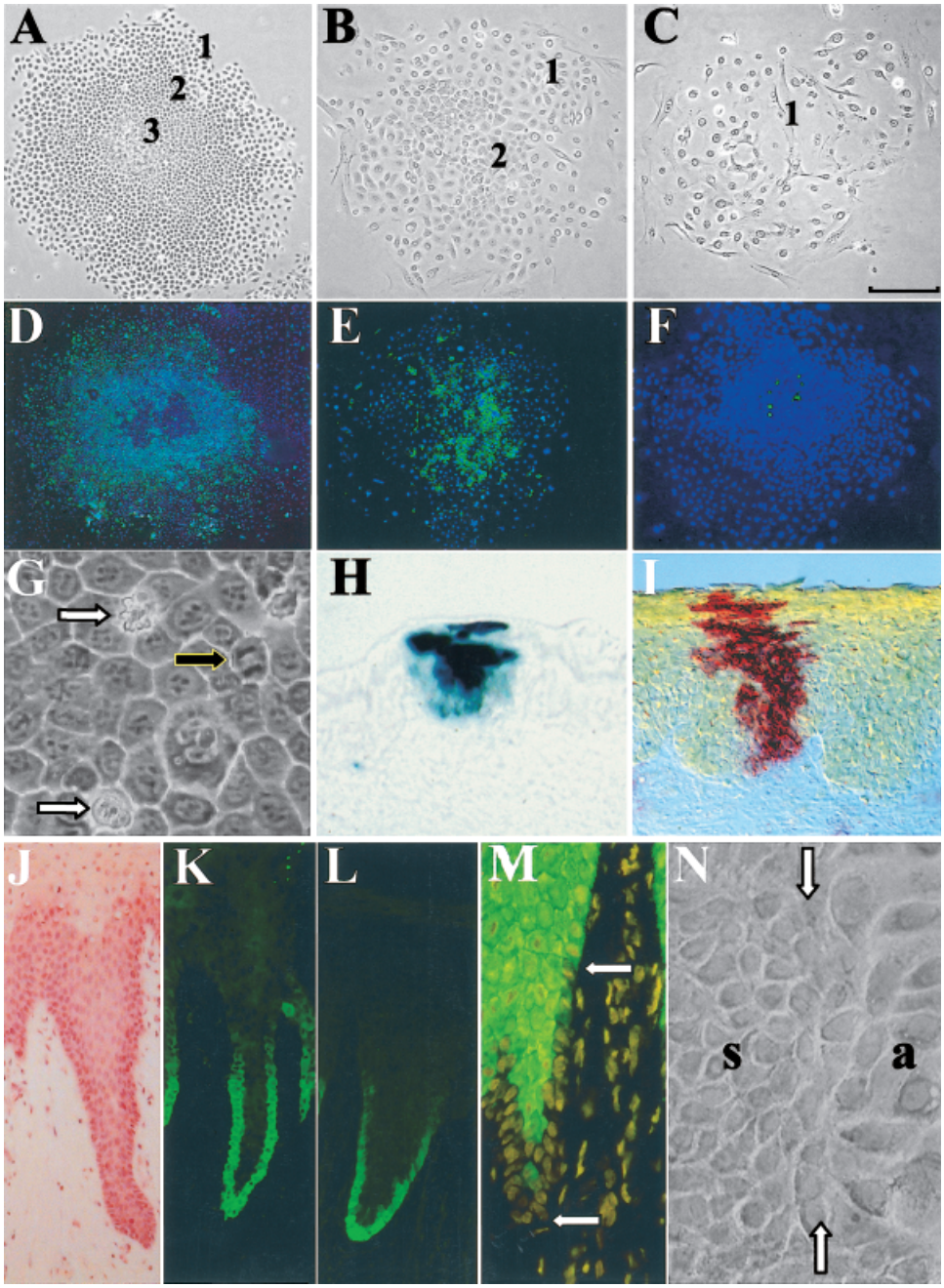

Figure 1
Colony morphologies formed by normal and malignant keratinocytes. Panels *A–C* show colonies developed from murine footpad keratinocytes 10 days after plating and photographed using phase contrast microscopy. Panel *A* shows a Type III colony consisting of a peripheral zone of large flattened Type 1 cells, an intermediate zone of smaller and more uniform Type 2 cells, and a central zone of small Type 3 cells. Panel *B* shows a Type II colony consisting of only Type 1 and 2 cells, and Panel *C* a Type I colony containing only Type 1 cells. Panels *D*, *E* show murine palatal Type III colonies, similar to that shown in Fig 1*A*, but stained by immunofluorescent methods with nuclei counter stained with Hoechst 333258. An antibody against cytokeratin 6 (*D*) shows staining of a zone corresponding to Type 2 cells and little staining of the zones of Type 1 and 3 cells. An antibody against cytokeratin 15 (*E*) shows staining of cells in the central region but with some staining of scattered cells in other regions. Panel *F* shows staining for Caspase 3 that is restricted to cells in the central zone. Panel *G* shows two blebbed cells (*white arrows*) corresponding to those stained for Caspase 3 in Panel D. This higher magnification phase contrast image of the central zone also shows a metaphase figure (*black arrow*). Panel *H* shows an organotypic culture, constructed of human skin fibroblasts and transduced keratinocytes, after transplantion to a SCID mouse for 12 wk. A small cluster of transduced cells that corresponds to a columnar unit of epidermal structure is seen. Panel *I* shows a similar preparation constructed from human palatal fibroblasts and transduced keratinocytes. A cluster of transduced cells runs through the full epithelial thickness but is narrower than the low rete ridges that have formed. Panels *J–L* show sections of normal human palatal mucosa. The section stained with hematoxylin and eosin (*J*) shows the appearance of the long regular rete that extend down into the connective tissue of the lamina propria. Panel *K* shows a section stained with an antibody against cytokeratin 19, reported to be a markers of stem cell zones; only basal cells of the deeper part of the rete are stained. Panel *L* shows a similar pattern of staining with an antibody against cytokeratin 15. Panel *M* shows part of a section stained with an antibody against cytokeratin 16, a marker of early mucosal differentiation. The arrows indicate a zone of unstained basal cells at the deep rete tip. Basal cells above the level of the upper arrow are strongly stained. Panel *N* shows a high-power phase-contrast image of the region of junction between Type 3 stem and Type 2 amplifying cells in a murine Type III colony. The arrows indicate the sharp zone of transition from one cell type to the other, a change thought to correspond to Transition 1 shown in Fig 2.

deep tips of the epithelial rete, their position in human esophageal mucosa and in murine palate.

To clarify the pattern of distribution of stem cells in normal palatal epithelia (Fig 1*J*), frozen tissue sections were stained with antibodies whose binding marks either putative stem cell zones or early cell differentiation. Keratin (K) 19, a keratin reported to be expressed in regions of hair follicles and of the interfollicular epidermis believed to harbour stem cells (Michel *et al*, 1996), stained basal cells at the deep tips of the epithelia rete with basal cells over the tips of the connective tissue papillae staining weakly or not at all (Fig 1*K*). Keratin 15, another keratin reported to mark stem cell zones (Cotsarelis *et al*, 1999), gave a similar pattern with staining only of basal cells in the deep parts of epithelial rete. Keratin 16, a marker of early differentiation in mucosal epithelia, stained almost all of cells of the epithelium with the only unstained, and therefore undifferentiated, basal cells lying in a small zone lying within the region stained for K19 or K15 (Fig 1*L*). These staining patterns indicate that the stem cell zones in palatal epithelium, like those of esophageal epithelium and palmar epidermis (Lavker and Sun, 1982; Seery and Watt, 2000), are located at the deep tips of the epithelial rete, a position clearly at variance with the distribution of the clonal units identified by lineage marking in reconstructed tissues. Likely explanations for this discrepancy are that the experimental system is unable to support full redevelopment of normal epithelial structure or that a 12 wk time period after transplantation is insufficient for it to redevelop. We and others have previously shown that the ability of adult murine epithelia to form rete structures, and also to develop more complex structures such as tongue papillae and hair, is dependent on interactions with the underlying mesenchyme (Mackenzie and Hill, 1984; Cunha *et al*, 1985; Oshima *et al*, 2001; Liang and Bickenbach, 2002). It is probable that the organotypic system fails to generate fully appropriate interactions, even when regionally appropriate fibroblasts are used to reconstruct tissues. It was interesting that even in this apparently inadequate system there was still redevelopment of clonal units, an observation providing further support for the conclusion that establishment of basic clonal patterns is an intrinsic epithelial property. The conflicting findings for lineage analysis and differentiation patterns indicate a need to re-evaluate assumptions about the validity of the lineage studies for murine and human epidermis. In both these epidermal tissues, however, clonal patterns correspond to units of structure and, for murine epidermis, there is independent evidence of centrally positioned stem cells (Potten, 1974; Ghazizadeh and Taichman, 2001). This seems to indicate the validity of clonal lineage patterns in thin epidermis but development of some alternative way of confirming this conclusion would be valuable.

Mechanisms Underlying Stem and Amplification Patterns

Figure 2 illustrates the type of stem and amplifying cell system hypothesized to be set up by normal keratinocytes both *in vivo* and *in vitro*. Three cell compartments (stem = S, amplifying = A, and post-mitotic differentiating = TD) are

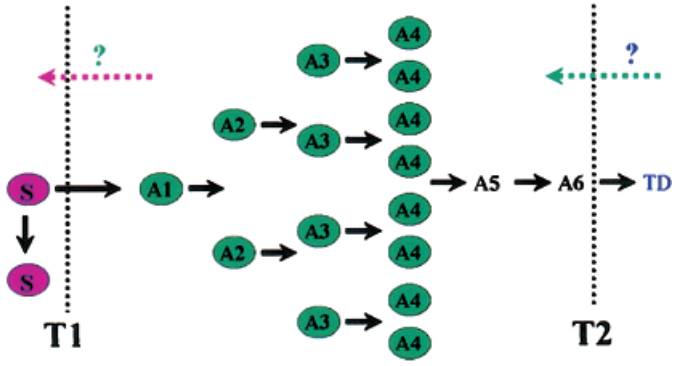

Figure 2
A diagrammatic representation of the cell divisions and transitions associated with maintenance of epithelial stem and amplification cell patterns. For the description, see text.

separated by two transitions T1 and T2, represented by dotted lines. In the normal steady state, each stem cell division produces, on average, one stem cell (S) and one amplifying cell (A1) that undergoes a series of amplifying divisions to produce terminally differentiating cells. In the diagram shown, five amplifying divisions produce 32 differentiating cells. It can be seen that the T2 transition controls the number of differentiated cells produced per stem division and that a shift of the T2 transition one tier to the left or right halves or doubles the number of cells produced. This will change the total number of cells in the population, and hence the proportion of stem cells within it, but changes of this transition will not directly influence the absolute number of stem cells. The size of stem cell territories, however, is expected to be related to their degree of amplification division, and thus likely to be controlled by the T2 transition, and there is evidence to suggest that the patterning, and hence size, of epidermal stem cell territories is associated with notch/delta interactions (Lowell *et al*, 2000).

An increase or decrease in the absolute number of stem cells will depend upon the proportion of stem cell progeny making the T1 transition following division. Lack of T1 transition S → A will lead to accumulation of stem cells and, conversely, increased transition S → A to stem cell loss. The molecular mechanisms and biochemical regulators of these transitions are not well established but over-expression of c-myc in transgenic mouse epidermis leads to stem cell depletion, possibly through enhancement of the T1 transition (Waikel *et al*, 2001). Similarly, targeted deletion of the p63 gene leads to an epidermal atrophy consistent with stem cell loss (Koster *et al*, 2002). It needs to be borne in mind, however, that little is known about the sharpness of this transition, i.e., its possible extension over more than a single division, or about the ability of cells to revert to the stem cell compartment having left it (Potten and Loeffler, 1990). In addition to their theoretical interest, these T1 mechanisms are likely to be of significance both to the mechanisms of tumor expansion and to the development of targeted therapies for the eradication of tumor stem cells. The inability to identify and to observe the fate of individual human stem cells *in vitro* has precluded direct investigation of factors influencing the T1 and T2 transitions. The extent to which this *in vitro* murine system provides information

directly relevant to the behavior of human stem cells remains to be demonstrated, but there seems to be a general similarity between the murine Type I, II, and III colonies and human paraclones, meroclones, and holoclones. The visible transition from Type 3 to Type 2 cells in murine colonies should enable investigation of changes in macromolecular expression that are associated with the T1 transition and, if the murine Type 3 cells do in fact correspond to stem cells, this system will provide a useful mechanism both for developing culture conditions conducive to extended stem cell expansion and for stem cell isolation for analysis of their molecular properties.

As illustrated in Fig 2, it is assumed that that in crossing the T1 boundary, stem cells become committed to differentiation but whether this is a irrevocable change, or as indicated by the red arrow, cells in positions A1 or A2 could return to the stem cell compartment is uncertain. Stem cells are assumed to be undifferentiated, in the sense that they are not committed to terminal differentiation, but there are intrinsic differences between epithelial stem cells of different regional origins and they normally appear stably committed to production of cells entering regionally restricted phenotypic pathways. It is also apparent that this commitment is retained during extensive passage *in vitro*. The role of other possible control mechanisms such as local microenvironmental influences producing a stem cell "niche" also remains unclear (Hall and Watt, 1989; Spradling *et al*, 2001). Stem cell spacing appears to be a basic epithelial property that is maintained during growth and regeneration (Mackenzie and Zimmerman, 1981; Mackenzie, 1997) and, in that sense, the stem cells exist within an epithelial niche of their own making, possibly controlled by delta/notch signaling (Lowell *et al*, 2000). By influencing the phenotypic commitment of a stem cell, however, and by influencing the degree of amplification undertaken by its progeny, the important mesenchymal contribution to the niche could be modulation of the intrinsic stem cell pattern into a range of new and more complex structures.

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